Adenosine Triphosphoric Acid as a Factor of Nervous Regulation of $Na^+/K^+/2Cl^-$ Cotransport in Rat Skeletal Muscle Fibers

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Exogenous adenosine triphosphoric acid produces a biphasic effect on the resting membrane potential of muscle fibers in rat diaphragm. Depolarization of the sarcolemma observed 10 min after application of adenosine triphosphoric acid results from activation of $Na^+/K^+/2Cl^-$ cotransport. The increase in chloride cotransport is related to activation of postsynaptic P2Y receptors and protein kinase C. Repolarization of the membrane develops 40 min after treatment with adenosine triphosphoric acid and after 50 min the resting membrane potential almost returns the control level. This increase in the resting membrane potential of the sarcolemma is probably associated with activation of the Na^+/K^+ pump and increase in membrane permeability for chlorine ions in response to long-term activity of Cl^- cotransport. Thus, adenosine triphosphoric acid co-secreted with acetylcholine in the neuromuscular synapse probably plays a role in the regulation resting membrane potential and cell volume of muscle fibers.

Key Words: *adenosine triphosphoric acid; P2Y receptors; chloride cotransport; resting membrane potential; neuromuscular synapse*

Cotransmitter adenosine triphosphoric acid (ATP) is co-secreted with the major neurotransmitter acetylcholine in the neuromuscular synapse [10]. It should be emphasized that the ATP/acetylcholine ratio in the synaptic vesicle of the nervous terminal is 1:6 [5,10]. Therefore, ATP concentration in the synaptic cleft during quantum secretion of this transmitter varies from 0.1 to 1.0 mmol/liter [14]. The effects of ATP in various organs and tissues are realized via 2 subtypes of specific receptors, including ionotropic P2X receptors and metabotropic P2Y receptors [8]. Much attention was paid to the effect of presynaptic P2Y receptor activation in the neuromuscular synapse. ATP plays a stimulatory or inhibitory effect on quantum secretion of the neurotransmitter, which is realized via these receptors [2]. However, ATP can produce a variety of effects in the neuromuscular synapse of mammals. Previous studies showed that neuromuscular synapses of chickens [1], frogs [4], and rats [7] contain not only presynaptic, but also postsynaptic P2Y receptors. Moreover, ATP is involved in the formation of the neuromuscular synapse and maintenance of its morphofunctional properties [11]. ATP potentiates postsynaptic fluxes in nicotinic receptors [4], prevents degradation of cholinoceptors [7], and modulates muscle contraction upon stimulation of the motor nerve [13]. Wide range of ATP functions in the neuromuscular apparatus suggests that this cotransmitter plays an important physiological role. However, ATP has a variety of functions.

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Here we studied possible role of this cotransmitter in nervous regulation of chloride cotransport in the muscle membrane.

MATERIALS AND METHODS

Experiments were performed on male albino laboratory rats weighing 150-200 g. The animals were decapitated under ether anesthesia. The diaphragmatic muscle was isolated in the costal arch. Diaphragmatic muscle fragments were continuously perfused with physiological saline for warm-blooded animals, which contained 120 mmol/liter NaCl, 5 mmol/liter KCl, 2 mmol/liter $CaCl_2$, 1 mmol/liter $MgCl_2$, 17 mmol/liter glucose, 1 mmol/liter $NaHPO_4$, and 24 mmol/liter $NaHCO_3$ (95% O_2 and 5% CO_2, pH 7.2-7.4). Experiments were performed with the following reagents (Sigma): ATP (100 μmol/liter), suramin (100 μmol/liter), reactive blue 2 (30 μmol/liter), Evans blue (100 μmol/liter), bumetanide (5 μmol/liter), chelerythrine (10 μmol/liter), 4-α-phorbol-12-myristate-13-acetate (PMA, 0.5 μmol/liter), verapamil (1 μmol/liter), ouabain (100 μmol/liter), βγ-methylene adenosine-5'-triphosphate (AMP-PCP, 100 μmol/liter), and 4,4'-diisothiocyanate-stilbene-2,2'-disulfonic acid (DIDS, 300 μmol/liter).

The resting membrane potential (RMP) was measured in the synaptic cleft with microelectrodes for recording of biological potentials. The location of the recording electrode in the synaptic cleft was verified by the appearance of miniature endplate potentials. The results were analyzed by Student's *t* test (parametric statistical method). The differences between two samples were significant at $p<0.05$.

RESULTS

ATP (100 μmol/liter) produced a biphasic effect on RMP of rat muscle fibers. ATP caused depolarization of the sarcolemma, which reached maximum by the 40th minute after application (8.7±0.3 mV, $p<0.001$). Membrane repolarization in muscle fibers was observed over the next 50 min. RMP significantly increased and did not return to the control level (Fig. 1).

We studied factors and mechanisms for muscle membrane depolarization under the influence of ATP. Stabilization of RMP was observed 20-30 min after the start of ATP application. Therefore, all measurements of RMP were performed by the 20th minute. The depolarizing effect of ATP was completely abolished by selective P2 receptor antagonist suramin (100 μmol/liter) [8] and noncompetitive selective P2 receptor antagonist reactive blue 2 (30 μmol/liter) [8] (Fig. 2). Addition of antagonists to the solution had little effect on RMP (data not shown). P2X receptor antagonist Evans blue (100 μmol/liter) was used to evaluate the subtype of receptors that mediate the effect of ATP (P2X or P2Y receptors) [8]. ATP was shown to decrease RMP of muscle fibers after treatment with this antagonist (Fig. 2). However, Evans blue caused depolarization of the sarcolemma ($p<0.001$, Fig. 2). Published data show that various antagonists of P2 receptors serve as inhibitors of ectoATPases [8]. We hypothesized that the effect of Evans blue is related to inhibition of the enzyme, which hydrolyzes ATP in the synaptic cleft. These changes should be accompanied by accumulation of ATP in the extracellular space, which causes membrane depolarization. We found that the effect of Evans blue is abolished by P2 receptor antagonist reactive blue 2 (Fig. 2). Specific P2X receptor agonist AMP-PCP (100 μmol/liter) was used to exclude possible involvement of these receptors in the effect of ATP [6]. RMP of the sarcolemma remained unchanged after treatment with this agonist ($p>0.05$, Fig. 2). Hence, the depolarizing effect of ATP on the sarcolemma is realized via P2Y receptors. However, the mechanism for depolarization remains unclear.

Our previous studies showed that depolarization due to denervation of the diaphragmatic muscle is associated with activation of $Na^+/K^+/2Cl^-$ cotransport [12]. In our experiments, depolarization is probably related to activation this pump. Chloride cotransport blocker bumetanide (5 μmol/liter) was used to confirm this hypothesis [9]. This agent caused slight hyperpolarization of the muscle fiber membrane ($p<0.001$, Fig. 3). However, ATP

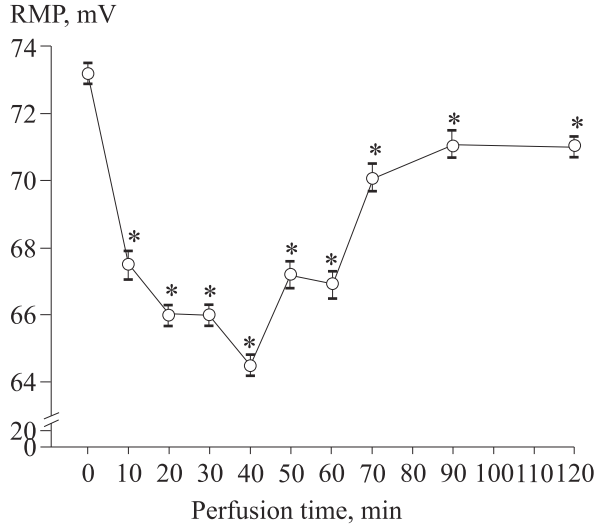

Fig. 1. RMP of muscle fibers in the presence of ATP. *$p<0.05$ compared to the control (0 min).

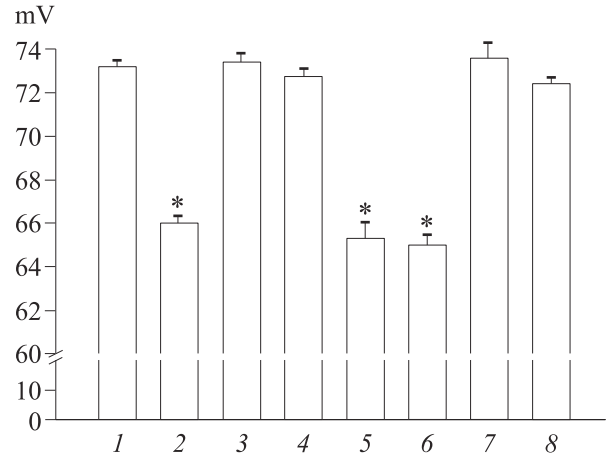

Fig. 2. RMP of control muscles (*1*) and muscle fibers in the presence of ATP (*2*), ATP+suramin (*3*), ATP+reactive blue 2 (*4*), ATP+Evans blue (*5*), Evans blue (*6*), Evans blue+reactive blue 2 (*7*), and AMP-PCP (*8*). Here and in Fig. 3: 20-min perfusion of muscles. **p*<0.05 compared to *1*.

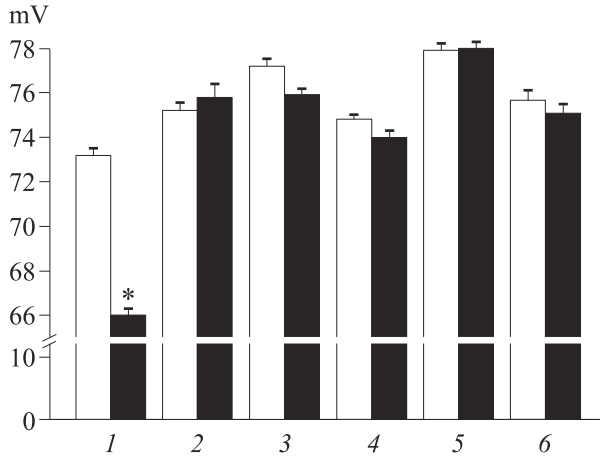

Fig. 3. RMP of muscle fibers under control conditions (light bars) and in the presence of ATP (dark bars): control muscles (*1*) and muscle fibers in the presence of bumetanide (*2*), chloride-free solution (*3*), chelerythrine (*4*), PMA (*5*), and verapamil (*6*). **p*<0.05 compared to the control (without ATP).

was ineffective after treatment with bumetanide ($p>0.05$, Fig. 3). This hypothesis is also confirmed by the results of experiments with modified Ringer's solution (substitution of Cl^- for SO_4^{2-}). Activation of $Na^+/K^+/2Cl^-$ cotransport does not occur in chloride-free solutions [9]. ATP was ineffective under these conditions ($p>0.05$, Fig. 3). These data confirm our hypothesis that ATP produces an activating effect on chloride cotransport.

The interaction of ATP with P2Y receptors is followed by activation of protein kinase C [8]. Moreover, the intensity of chloride cotransport is regulated by this enzyme [9]. Specific protein kinase C inhibitor chelerythrine (10 μmol/liter) [3] did not modulate RMP of the sarcolemma ($p>0.05$, Fig. 3), but prevented the depolarizing effect of ATP ($p>0.05$, Fig. 3). Protein kinase C activator PMA (0.5 μmol/liter) caused hyperpolarization of muscle fibers ($p<0.001$, Fig. 3). However, ATP had no effect on the membrane potential after treatment with PMA ($p>0.05$, Fig. 3). The effect of ATP was probably realized via Ca^{2+} channels, since calcium channel blocker verapamil (1 μmol/liter) abolished the depolarizing effect of ATP ($p>0.05$, Fig. 3).

RMP of muscle fibers progressively increased after 40-min perfusion of the neuromuscular preparation with ATP-containing solution, but remained unchanged by the 90th minute of application. It can be hypothesized that the increase in the sarcolemmal potential is related to a decrease in the intensity of chloride cotransport. However, treatment with bumetanide in the presence of ATP (90-min exposure of muscle fibers in the working solution) was followed by greater increase in RMP of the sarcolemma (by 5.1±0.4 mV, $p<0.001$, Fig. 3) as compared to RMP of muscle fibers after ATP perfusion. Therefore, chloride cotransport in muscle fibers remains unchanged by the 90th minute of treatment with ATP. The question arises: what is the cause for the increase in RMP under constant activity of chloride transport? Long-term transport should be followed by significant changes in the intracellular concentration of transported ions. It mainly concerns Na^+ and Cl^-, since cell membrane permeability to K^+ is relatively high under resting conditions. The rise in the intracellular concentration of Na^+ and Cl^- contributes to activation of the Na^+/K^+ pump and/or increase in membrane permeability to Cl^-. Strong evidence exists to support both assumptions. The depolarizing effect of ATP was partially restored in the presence of Na^+/K^+ ATPase inhibitor ouabain (100 μmol/liter). RMP decreased by 3.8±0.4 mV under these conditions ($p<0.001$ compared to the membrane potential of ouabain-perfused muscle fibers). Similarly to ouabain, ATP had a depolarizing effect (2.4±0.5 mV, $p<0.001$) after treatment with chloride channel blocker DIDS (300 μmol/liter) [9]. Besides the influence on chloride cotransport, long-lasting application of ATP has an indirect modulatory effect on the Na^+/K^+ pump and membrane chloride permeability.

We conclude that synaptic ATP activates P2Y receptors on the postsynaptic membrane, which is followed by an increase in $Na^+/K^+/2Cl^-$ cotransport in muscle fibers. This effect of ATP is mediated by protein kinase C and depends on the presence of Ca^{2+}. Our previous experiments showed that nonquantal secretion of acetylcholine and glutamate from the nerve ending to the motor synapse serves as a natural process of nervous regulation for chlo-

ride cotransport [12]. In the present study we revealed the existence of another factor, which is involved in this regulatory process. Since non-quantal acetylcholine and glutamate inhibit chloride transport, ATP produces an opposite effect. The opposite effects of these factors are probably required for fine regulation of chloride cotransport. Published data show that this transport is involved in potential formation and regulation of the cell volume [9,12]. Hence, ATP is also involved in these processes. Moreover, the influence of ATP on the neuromuscular apparatus is of considerable biological significance. ATP-induced depolarization is followed by a decrease in the excitation threshold of muscle fibers. The reliability of synaptic transmission increases under these conditions. This mechanism probably contributes to facilitation of excitation transmission in the neuromuscular synapse under the influence of endogenous ATP.

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REFERENCES

1. R. C. Coi, M. L. Man, K. K. Ling, *et al*., *J. Neurosci*., **21**, No. 23, 9224-9234 (2001).
2. R. A. Cunha and J. A. Ribero, *Life Sci*., **68**, No. 2, 119-137 (2000).
3. A. E. Eckly-Michel, A. Le Bec, and C. Lugnier, *Eur. J. Pharmacol*., **324**, No. 1, 85-88 (1997).
4. W. M. Fu, Y. H. Chen., K. F. Lee, and J. C. Liou, *Eur. J. Neurosci*., **9**, No. 4, 676-685 (1997).
5. M. Israel and Y. Dunant, *Mol. Neurobiol*., **16**, No. 1, 1-20 (1998).
6. S. E. O'Connor, B. E. Wood, and P. Leff, *Br. J. Pharmacol*., **101**, No. 3, 640-644 (1990).
7. J. O'Marlley, C. T. Moore, and M. M. Salpeter, *J. Cell Biol*., **138**, No. 1, 159-165 (1997).
8. V. Ravelic and G. Burnstock, *Pharmacol. Rev*., **50**, No. 3, 413-492 (1998).
9. J. M. Russell, *Physiol. Rev*., **80**, No. 1, 211-276 (2000).
10. E. M. Silinsky and R. S. Redman, *J. Physiol*., **492**, Pt. 3, 815-822 (1996).
11. K. Tsim and E. A. Barnard, *Neurosignals*, **11**, No. 1, 58-64 (2002).
12. A. Kh. Urazaev, N. V. Naumenko, E. E. Nikolsky, and F. Vyskocil, *Neurosci. Res*., **33**, No. 2, 81-86 (1999).
13. E. S. Vizi, K. Nitahara, K. Sato, and B. Sperlagh, *J. Auton. Nerv. System*, **81**, Nos. 1-3, 278-284 (2000).
14. H. Zimmermann, *Trends Pharmacol. Sci*., **20**, No. 6, 231-236 (1999).